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Genetic Relatedness, Antimicrobial and Biocide Susceptibility Comparative Analysis of Methicillin-Resistant and -Susceptible *Staphylococcus pseudintermedius* from Portugal

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Forty methicillin-resistant and -susceptible *Staphylococcus pseudintermedius* (MRSP and MSSP, respectively) from colonization and infection in dogs and cats were characterized for clonality, antimicrobial, and biocide susceptibility. MSSP were genetically more diverse than MRSP by multi-locus sequence typing and pulsed-field gel electrophoresis. Three different *spa* types (t06, t02, t05) and two SCC*mec* types (II-III and V) were detected in the MRSP isolates. All MRSP and two MSSP strains were multidrug-resistant. Several antibiotic resistance genes (*mecA*, *blaZ*, *tet*(M), *tet*(K), *aac(6')-Ie–aph(2')-Ia*, *aph(3')-III*, *ant(6)-Ia*, *sat4*, *erm*(B), *lnu*(A), *dfr*(G), and cat_{pC221}) were identified by microarray and double mutations in the *gyrA* and *grlA* genes and a single mutation in the *rpoB* gene were detected by sequence analysis. No differences were detected between MSSP and MRSP in the chlorhexidine acetate (CHA) minimum inhibitory concentrations (MICs). However, two MSSP had elevated MIC to triclosan (TCL) and one to benzalkonium chloride and ethidium bromide. One MSSP isolate harboured a *qacA* gene, while in another a *qacB* gene was detected. None of the isolates harboured the *sh-fabI* gene. Three of the biocide products studied had high bactericidal activity (Otodine®, Clorexyderm Spot Gel®, Dermocanis Piocure-M®), while Skingel® failed to achieve a five log reduction in the bacterial counting. *S. pseudintermedius* have become a serious therapeutic challenge in particular if methicillin- resistance and/or multidrug-resistance are involved. Biocides, like CHA and TCL, seem to be clinically effective and safe topical therapeutic options.

Introduction

Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) has emerged recently and has become a serious therapeutic challenge for veterinarians, due to multidrug resistance.[9,12,24,30] They are a major cause of skin and urinary tract, and hospital acquired infections in dogs and cats.[12,30] Originally, two major MRSP clones were found to spread in Europe (ST71-t02-SCC*mec* II-II) and North America (ST68-t06-SCC*mec* V).[4,24,30] Although more recent reports have yet identified other *S. pseudintermedius* lineages carrying the *mecA* gene,[11,22] methicillin-susceptible *S. pseudintermedius* (MSSP) tend to be genetically more diverse than MRSP.[2,4]

In addition to the *mecA* gene, MRSP isolates usually have mutations in the gyrase and topoisomerase genes, conferring resistance to fluoroquinolones[10] and several other genes, which mediate resistance to gentamicin, kanamycin, erythromycin, clindamycin, streptomycin, tetracycline and trimethoprim.[12,30] Resistance to rifampicin and chloramphenicol has also been reported in some MRSP strains.[12,17,30] This pattern of multidrug resistance is normally in contrast to what happens with MSSP.[12] Resistance to ampicillin and penicillin is often reported in MSSP isolates, but they are usually susceptible to the other antimicrobial classes.[12] Yet, treatment of MRSP infections is based on the same principles as MSSP infections, usually involving systemic and/or topic therapy. The difference lies on the number of antimicrobial options available for a successful treatment. While there are several antimicrobial options for MSSP therapy, some of the antimicrobials used for the treatment of MRSP infections are not licensed for veterinary use and considered "critically important" for human medicine by the World Health Organization.[32] Other antimicrobials, like rifampicin and

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chloramphenicol, are potentially toxic and have suboptimal pharmacological features for small animals.[12,17] For this reason, topical therapy, especially antiseptic preparations, has gained a renewed interest. Biocide topical therapy can be used as solo or as an adjuvant for the treatment of skin, ear and wound infections.[12] Previous studies have assessed the *in vitro* efficacy of biocides through determination of minimum inhibitory concentrations (MICs) and/or minimum bactericidal concentrations.[15,27,33] Although the determination of MICs is important for the detection of efflux phenotypes (especially through detection of the ethidium bromide [EtBr] MICs) the *in vitro* efficacy of a biocide, as recommended by the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR), should involve the use of a neutralising agent or by the removal of the biocide.[25] This is important to avoid an over-estimation of the lethality of the biocide compound, since biocides are usually in contact with the bacteria only for a brief period of time.

To date, only a few studies have determined biocide susceptibility of *S. pseudintermedius*. This study compares the clonality, antimicrobial and biocide susceptibility of MSSP and MRSP that have been isolated from the nasal cavities of healthy animals as well as from infection sites.

Materials and Methods

Strain collection

Twenty MRSP and twenty MSSP strains isolated between 2007 and 2011 were included in the study. The isolates were collected at the Laboratory of Antimicrobial and Biocide Resistance, FMV-UTL, which receives samples from the Veterinary Teaching Hospital of FMV-UTL and private practices covering the area of the Lisbon region. Five isolates were from cats and 35 were from dogs. These included clinical infection (urinary tract infection, $n=6$; skin infection, $n=10$; ear infection, $n=5$; surgical site infection, $n=1$) and nasal colonization isolates ($n=18$).

Multi-locus sequence typing, spa *and SCC*mec *typing*

Isolates were characterized by Multi-locus sequence typing (MLST) using the MLST scheme of Bannoehr *et al.*,[2] which is based on five housekeeping genes (*pta*, *cpn60*, *tuf*, 16S rRNA and *agrD*),[24] and also by the newly described *S. pseudintermedius* MLST scheme, which is based on seven housekeeping genes (*ack*, *cpn60*, *fdh*, *pta*, *purA*, *sar*, *tuf*).[27] MRSP isolates were also characterized by *spa* and SCC*mec*-typing. *spa*-typing was performed by sequencing the polymorphic region of protein A gene (*spa*) and *spa* types were assigned according to previously proposed guidelines.[24] SCC*mec* types were determined using the multiplex PCR 1 and the multiplex PCR 2 according to Kondo and collaborators.[18] In multiplex PCR 1, the presence of *mecA* was confirmed and the *ccr* gene complex was determined. In multiplex PCR 2, the *mec* class complex was assessed.[18] The combination of the type *ccr* and *mec* complex was used to consign SCC*mec* types. *SCCmec* II-III was identified by PCR using primers described previously.[23]

eBURST analysis

Predicted lines of evolutionary descent in our collection of MRSP and MSSP isolates were identified using the eBURST algorithm (http://eburst.mlst.net). eBURST identified groups of related sequence types (ST) by assigning all members that shared identical alleles at four of the five gene loci (MLST-5 scheme) or six of the seven gene loci (MLST-7 scheme) with at least one other member of the group.[2] The founding ST of each group was determined by the ST with the greatest number of single locus variants (SLV).[28] Subgroups were defined by the existence of at least three SLV.

Pulsed-field gel electrophoresis

The *S. pseudintermedius* strains were compared for their genetic relatedness by *Sma*I macrorestriction, using a previously described protocol.[9] The *Sma*I fragment patterns were analysed with BioNumerics (Applied Maths, Kortrijk, Belgium), the similarities between profiles were calculated using the Dice coefficient with a maximum position tolerance of 1.0%. The patterns were clustered by using the unweighted pair group method with arithmetic averages based on a similarity cut-off value of 80%.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was determined by the disk diffusion method and according to the Clinical and Laboratory Standards Institute guidelines.[7] The antibiotic resistance genes were detected using the custom-made microarray AMR+ve-2 (Alere GmbH, Cologne, Germany)[24] and by PCR.[19,24] Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* and *grlA* were determined by PCR using the following primers: gyrA_pseudFW 5′-ATGAGTG TTATCGTATCTCGTGC-3′, gyrA_pseudRV 5′-GAACCGAA GTTACCTTGACCAT-3′, grlA_pseudFW 5′-AATACGTAT GATAAACATTTTCG-3′ and grlA_pseudRV 5′-TCGGTAT-CATCATAGTTCGG-3′, respectively. Mutations in the rifampicin resistance-determining region (RRDR) within the *rpoB* gene of the rifampicin-resistant isolates were amplified by PCR and sequenced as described previously.[17]

Biocide susceptibility

MICs were determined for the following antiseptics and dye: chlorhexidine acetate (CHA), benzalkonium chloride (BAC), triclosan (TCL) and EtBr. EtBr MIC determination is a simple screening procedure for identifying strains, which have increased expression of efflux pump genes or an efflux phenotype.[8] CHA, BAC and TCL were determined to further characterize any efflux phenotype. The bactericidal activity (at 5 minutes and 20°C) of four commercial dermatological preparations (Otodine®, Clorhexyderm Spot Gel®, Dermocanis Piocure-M® and Skingel®) was determined against MRSP and MSSP according to the document NF EN 1040–Essai quantitatif de suspension pour l'évaluation de l'activité bactericide de base des antiseptiques et des désinfectants chimiques.[1] Both Otodine and Clorhexyderm Spot Gel contain CHA (0.15% and 0.3%, respectively), Dermocanis Piocure-M has TCL (0.3%) and Skingel contains zinc oxide (10%). The full formulation of the biocide products can be found at the manufacture's website (www.icfpet.it). Briefly, isolates were grown on a solid medium for 24 hours at 37°C and suspended on a diluent to $1.5–5\times10^8$ colony-forming units/ml. Each cell suspension was inoculated into water containing the biocides and was exposed for 5 minutes, as recommended by NF1040.

To inactivate the biocides, the bacteria-biocide mixture was transferred into the neutralization medium (phosphate buffer 0.25 mmol/L pH 7.2) for 5 minutes. Then the mixture was inoculated onto Tryptone-Soy agar plates without the biocides. Bacteria growth was observed after incubation at 37°C for 24 hours. Bactericidal activity was defined as a logarithmical reduction on bacterial cell counts of at least five logarithms. *S. aureus* ATCC 29213 and *S. aureus* ATCC 6538 were used as quality controls. The detection of efflux genes *qacA/B*, *smr*, *qacG*, *qacH* and *qacJ* was performed by PCR.[3,8] The *qacA/B* positive amplicons were sequenced. The detection of *sh-fabI* was performed by PCR using primers described recently by Ciusa *et al.*, using *S. aureus* strain M0091 as a positive control.[6]

Results

Strain characterization by genotyping

The epidemiological, genotypic and phenotypic traits of the forty MRSP and MSSP isolates under study are shown in Table 1. MLST-7 allowed a better discrimination than MLST-5 and further distinguished among strains (Table 1). The MSSP strains were divided into 19 or 24 different STs according to the MLST-5 and MLST-7 schemes, respectively (see Table 1). Two novel *cpn60* alleles (alleles 43 and 44, accession numbers JX976294 and JX982108, respectively) and four novel *pta* alleles (alleles 32, 34, 35 and 36, accession numbers JX982110, KC438371, JX982112 and JX987962, respectively) were found. Using the MLST-5 scheme, 17 MRSP belonged to ST71, two belonged to ST97 and one to ST2 (Fig. 1a). When applying the new MLST-7 scheme only 14 MRSP ST71 isolates were assigned to the ST71, and three being assigned to ST203; two ST97 were subdivided into ST196 and ST213, and ST2 was assigned to ST195. Yet ST203 and ST195 belonged to the clonal complex (CC) 71, as detected by the eBURST analysis (Fig. 1b). Likewise ST196 and ST213 differed only by one allele and belonged to CC196 (Fig. 1b). Pulsed-field gel electrophoresis (PFGE) analysis, based on a similarity cut-off value of 80%, revealed two major clusters of MRSP, one containing the CC71 isolates and the other cluster having the CC196 strains (Fig. 2). The MRSP isolate ST195 was non-typeable by SmaI restriction PFGE. Similar to MLST results, PFGE analysis revealed that the MSSP isolates were genetically more diverse (Fig. 3). eBURST analysis performed in our collection of MRSP and MSSP isolates was very different when using the MLST-5 and MLST-7 schemes. When applying the MLST-5 scheme eBURST showed that the *S. pseudintermedius* isolates belonged to very similar STs, only differing in one or two of the five loci examined (Fig. 3a). As expected, eBURST analysis using the MLST-7 scheme had very different results, with only a few STs relating with another (Fig. 1b) and the MSSP being singletons (data not shown).

Antimicrobial susceptibility and resistance genes

Antimicrobial resistance patterns of the *S. pseudintermedius* isolates are shown in Table 1. All MRSP isolates were resistant to erythromycin, clindamycin, fluoroquinolones (ciprofloxacin, enrofloxacin, moxifloxacin, norfloxacin, ofloxacin and pradofloxacin), trimethoprim/sulfamethoxazole, gentamicin, tobramycin, kanamycin and streptomycin. Additionally 17 MRSP isolates had tetracycline-resistance, one had chloramphenicol resistance and one was resistant to rifampicin. MSSP were more susceptible than MRSP strains to the tested antibiotics. Eight strains were susceptible to all antibiotics. All isolates were susceptible to fluoroquinolones. Ampicillin and penicillin resistance was present in nine MSSP strains, while eight were resistant to tetracycline. Two MSSP strains were resistant to erythromycin, clindamycin, kanamycin, streptomycin and chloramphenicol and one was resistant to trimethoprim/sulfamethoxazole. Resistances were attributed to the presence of the penicillin binding protein gene *mecA* (all β-lactams), the β-lactamase gene *blaZ* (penicillin), the tetracycline resistance genes *tet*(M) and *tet*(K), aminoglycoside acetyltransferase and phosphotransferase gene *aac(6′)-Ie–aph(2′)-Ia* (all aminoglycosides except streptomycin), phosphotransferase gene *aph(3′)-III* (kanamycin, neomycin, paromomycin, amikacin, gentamicin B), streptomycin adenyltransferase gene *ant(6)-Ia*, the macrolide, lincosamide and streptogramin B 23S rRNA methylase gene *erm*(B), the lincosamide nucleotidyltransferase *lnu*(A), the chloramphenicol acetyltransferase gene cat_{pC221}, the trimethoprim-resistance dihydrofolate reductase gene *dfr*(G). Two amino acid substitutions (S84L of GyrA and S80I of GrlA) were found in the QRDR of fluoroquinolone-resistant isolates. The rifampicin-resistant isolate had an A522D substitution in the RRDR. One MSSP strain harboured a *lnu*(A) gene but was not resistant to clindamycin.

Biocide susceptibility, qac *and* sh-fabI *genes*

Fourteen and six MRSP isolates presented an MIC of 1 mg/L and 2 mg/L of BAC, respectively (Table 2). Eighteen MSSP isolates had an MIC of 0.5 mg/L to BAC (Table 2). All MRSP and MSSP strains had an MIC of 1 mg/L to CHA. All MRSP isolates and 18 MSSP had an MIC to TCL of ≤ 0.003 mg/L, while one isolate had an MIC of 0.125 mg/L. None of the isolates carried the recently described TCL resistance gene *sh-fabI*. MIC to EtBr were ≤ 4 mg/L, except for one isolate, which showed an EtBr MIC of 32 mg/L (Table 2). This MSSP isolate (FMV20A/08) had an MIC of four to BAC and harboured the quaternary compound resistance gene *qacA*. Another MSSP isolate (FMV750/10) had the *qacB* gene but no detectable efflux mechanisms. All MRSP isolates were negative for the efflux genes tested.

Three preparations, Otodine, Clorexyderm Spot Gel and Dermocanis Piocure-M, had bactericidal activity against all MRSP and MSSP isolates. However, Skingel could not achieve a five log reduction of the bacterial count.

Discussion

Methicillin resistance have only been recently reported in *S. pseudintermedius* strains, but their capacity to resist to antimicrobial therapy is already a worldwide concern.[23,30] ST71 was the predominant clone emphasizing its spread. The use of the new MLST scheme based on seven housekeeping genes allowed distinguishing between some of the strains of the CC71, revealing new ST195 and ST203. ST71 has been previously described among MRSP colonization isolates from dogs in Portugal;[9] however, this is the first report of MRSP ST196 and ST213 (CC196), which are not related to CC71. MSSP isolates, instead, were more genetically diverse, with all MSSP isolates corresponding to a single ST. These findings are in agreement with two previous reports,[2,4] where MRSP isolates were restricted to a small number of ST, while MSSP strains revealed substantial clonal diversity.

Table 1. Epidemiological, Genotypic, and Phenotypic Traits of the Forty Strains Included in the Study

Strains	*Sample source*	*Animal clinical status*	*ST based on 5-MLST*	*ST based on 7-MLST*	spa *type*	*SCCmec type*	*Antimicrobial resistance phenotype*
FMV1879B/07	Cat	Urinary tract infection	97	196	t06	V	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV5/08	Dog	Healthy	97	213	t06	V	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV29/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV34C/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV34D/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV67/08	Dog	Healthy	71	203	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV71/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV104/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV116/08	Dog	Healthy	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV178/09	Dog	Healthy	71	203	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV4877/10	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMVP61/ZP	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV3891/09	Cat	Urinary tract infection	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV1860/10	Cat	Otitis	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV3008/10	Dog	Otitis	71	203	t06	II-III	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-CIP-FQ
FMV3607/10	Cat	Urinary tract infection	71	71	t06	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ-CHL
FMV5819/10	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ-RIF
FMVStaph4	Dog	Pyoderma	71	71	t02	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV981/11	Dog	Pyoderma	71	71	t05	II-III	OXA-PEN-AMP-TET-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV13/2011	Dog	Discospondylitis	2	195	t02	II-III	OXA-PEN-AMP-ERY-CLI-GEN-TOB-KAN-STR-SXT-FQ
FMV5699/07	Dog	Urinary tract infection	169	207	–	–	–
FMV9/08	Dog	Healthy	40	17	–	–	–
FMV12/08	Dog	Healthy	52	216	–	–	PEN-AMP-ERY-CLI-KAN-STR-CHL
FMV15/08	Dog	Healthy	54	210	–	–	–
FMV20A/08	Dog	Healthy	93	211	–	–	PEN-AMP
FMV33/08	Dog	Healthy	192	201	–	–	–
FMV41/08	Dog	Healthy	176	212	–	–	TET
FMV52/08	Dog	Healthy	136	217	–	–	–
FMV66/08	Dog	Healthy	171	214	–	–	TET
FMV76/08	Dog	Healthy	177	215	–	–	PEN-AMP-TET
FMV750/10	Dog	Pyoderma	29	200	–	–	PEN-AMP-TET
FMV2944/10	Dog	Pyoderma	17	202	–	–	PEN-AMP-TET
FMV635/10	Dog	Urinary tract infection	170	209	–	–	PEN-AMP-TET
FMV3413/09	Dog	Otitis	20	204	–	–	TET
FMV2183/10	Dog	Otitis	172	197	–	–	–
FMV2999/10	Dog	Urinary tract infection	40	199	–	–	–
FMV2218/10	Dog	Pyoderma	44	198	–	–	–
FMV5386/10	Dog	Pyoderma	174	206	–	–	PEN-AMP
FMV6098/10	Dog	Pyoderma	175	208	–	–	PEN-AMP-SXT
FMV4778/09	Cat	Otitis	178	205	–	–	PEN-AMP-TET-ERY-CLI-KAN-STR-CHL

AMP, ampicillin; CHL, chloramphenicol; CLI, clindamycin; ERY, erythromycin; FQ, fluoroquinolones, including ciprofloxacin, enrofloxacin, moxifloxacin, norfloxacin, ofloxacin, and pradofloxacin; GEN, gentamicin; KAN, kanamycin; OXA, oxacillin; PEN, penicillin; RIF, rifampicin; TET, tetracycline; TOB, tobramycin; ST, sequence types; STR, streptomycin; SXT, sulfamethoxazole/trimethoprim.

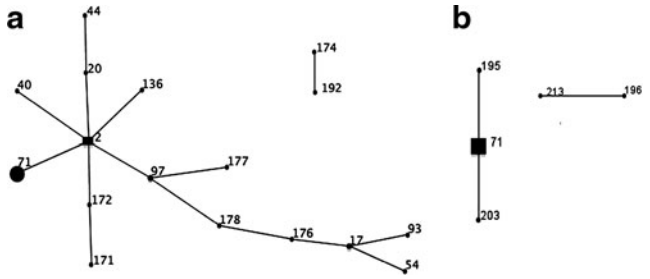

FIG. 1. Schematic diagram of the clonal relatedness of *Staphylococcus pseudintermedius* sequence types (ST) predicted by eBURST analysis using the multilocus sequence typing (MLST)-5 **(a)** and the MLST-7 **(b)** schemes, respectively. Each black dot represents an ST and the dot size is proportional to the number of isolates of that ST. The square corresponds to the predicted group founder and ST97 and ST17 represent predicted subgroup founders **(a)**. Single-locus variants are linked by lines. Methicillin-susceptible *S. pseudintermedius* (MSSP) singletons are not shown in this figure.

However, two recent reports found the *mecA* gene in a considerable high number of ST,[11,22] indicating that different lineages of *S. pseudintermedius* can acquire SCC*mec* elements. Only two types of SCC*mec* elements were detected among the MRSP from Portugal, namely SCC*mec* II-III in isolates of CC71 and SCC*mec* V in isolates of CC196.

Antimicrobial resistance is typically very different between MRSP and MSSP. While MRSP tend to be multidrug-resistant, MSSP are usually only resistant to ampicillin and penicillin, due to the presence of the *blaZ* gene.[12] Accordingly we found a multidrug resistant pattern in all MRSP isolates. However, two MSSP strains were also resistant to more than three antimicrobial classes, categorizing these strains as multidrug-resistant. Some studies have also identified multidrug-resistance among MSSP isolates.[13,14,29] Nevertheless, in the majority of the studies MSSP strains were only resistant to ampicillin and one additional antimicrobial class.[12,30] Several antimicrobial resistance genes have been detected in *S. pseudintermedius* strains[16] and our strains exhibit the same genes as detected before. Contrary to the study of Vanni and colleagues,[31] which only detected resistance to second- and third-generation fluoroquinolones in *S. pseudintermedius* isolates, our fluoroquinolone-resistant *S. pseudintermedius* strains were resistant to second (ciprofloxacin, enrofloxacin, norfloxacin, and ofloxacin), third generation (pradofloxacin), and fourth generation (moxifloxacin) fluoroquinolones. The same authors argued that a single alteration in *grlA* would be sufficient to confer resistance against older fluoroquinolones but an additional mutation in *gyrA* was required for resistance to new fluoroquinolones to develop, as it occurs in *S. aureus* and coagulase-negative staphylococci isolates.[31] Accordingly, our strains presented resistance to all the fluoroquinolones tested, including moxifloxacin, due to the presence of mutations at both the *gyrA* and *grlA* genes.

Surprisingly there was a major difference between the mechanisms of resistance to tetracycline: *tet*(K) genes were only identified among MRSP strains, while *tet*(M) was only found among MSSP isolates. The *tet*(K) gene codes for an efflux pump of the major facilitator superfamily and is usually found on small plasmids.[16] In contrast, *tet*(M) codes for ribosome protective proteins and has been identified as part of conjugative transposons, such as Tn*916* and Tn*1545*.[16] Other studies have identified *tet*(M) in other MRSP strains, yet in MRSP ST71 only *tet*(K) has been detected, which could indicate that this clone has a preference for plasmid-borne tetracycline resistance rather than *tet* transposon-borne genes.

Rifampicin resistance in MRSP isolates has been described previously.[17] Mutation at *rpoB* codon 522 was identified in a clinical isolate after treatment of a clinical infection with a combination of rifampicin and tetracycline.[17] Our rifampicin-

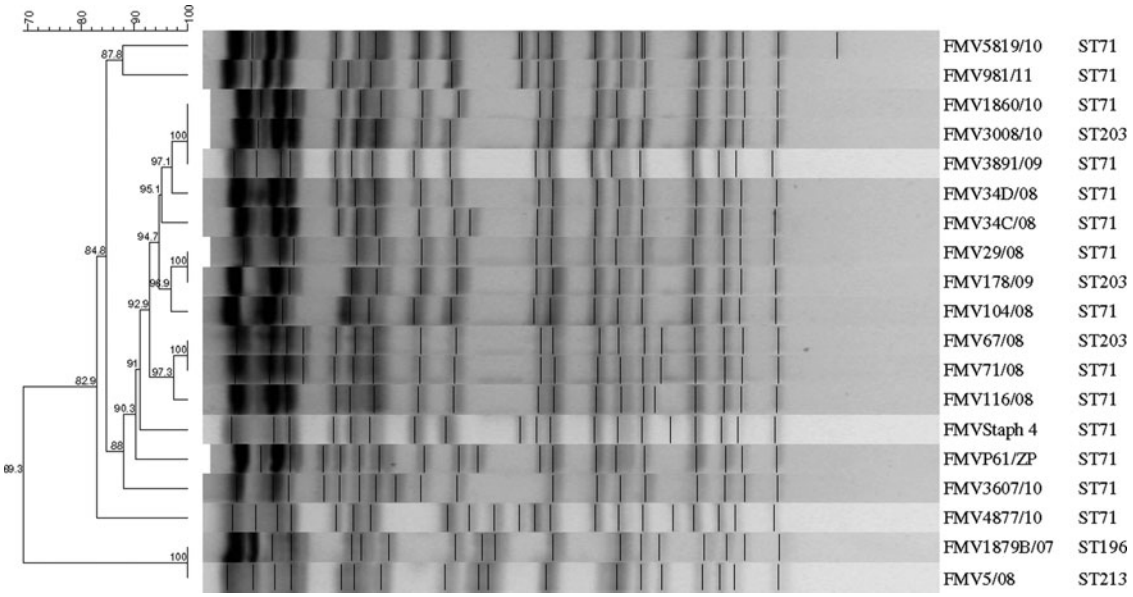

FIG. 2. Dendogram of chromosomal DNA digested with SmaI of methicillin-resistant *S. pseudintermedius* strains and relatedness to ST. Pulsed-field cluster determination using a Dice similarity coefficient with an optimization of 1% and a band tolerance setting of 1%.

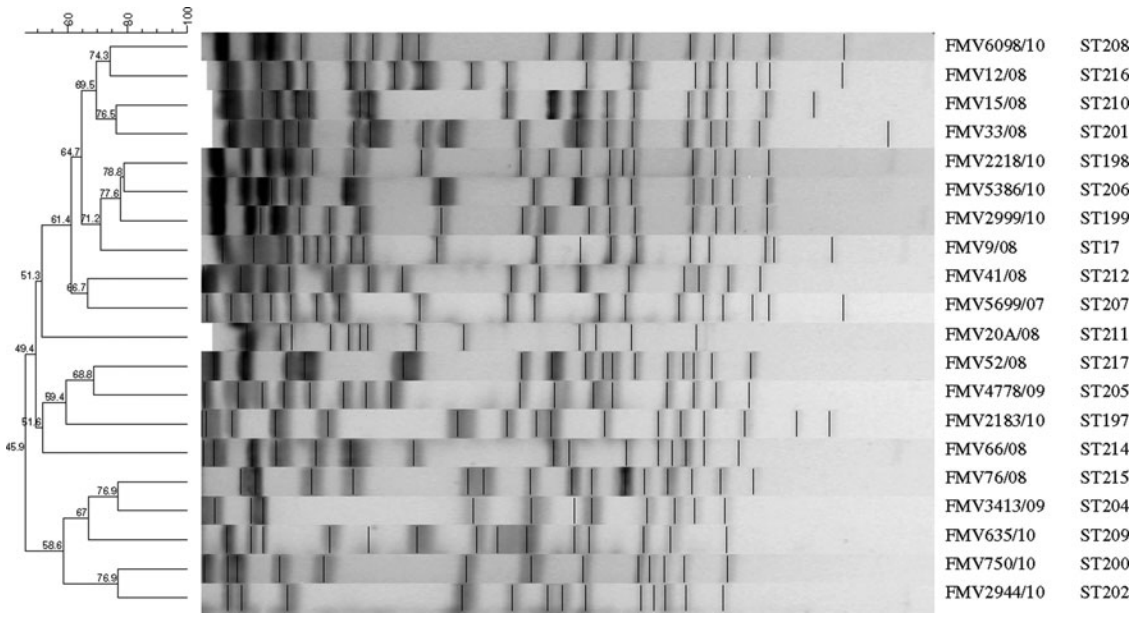

FIG. 3. Dendogram of chromosomal DNA digested with SmaI of MSSP strains and relatedness to ST. Pulsed-field cluster determination using a Dice similarity coefficient with an optimization of 1% and a band tolerance setting of 1%.

resistant MRSP isolate came from a dog with pyoderma, with no previous recorded history of rifampicin treatment. As stated before,[17] the observed *rpoB* mutation could have occurred spontaneously or the isolate could have been transferred from a previously treated dog.

Since the discovery of multidrug-resistant *S. pseudintermedius* there has been an increasing interest in additional bactericidal therapeutics, other than the use of antibiotics. Susceptibility to biocides has now become an urgent matter.

Table 2. MICs of Dyes (Ethidium Bromide) and Biocides (Benzalkonium Chloride, Chlorhexidine Acetate and Triclosan), and Genes Associated with Efflux Phenotype

	MICs (mg/L)				
Number of strains	*BAC*	*CHA*	*EtBr*	*TCL*	*Efflux genes*
MRSP					
10	1	1	2	≤0.003	–
3	2	1	4	≤0.003	–
3	2	1	2	≤0.003	–
2	1	1	4	≤0.003	–
1	1	1	1	0.007	–
1	1	1	2	0.007	–
MSSP					
11	0.5	1	1	≤0.003	–
6	0.5	1	2	≤0.003	
1	4	1	32	0.125	*qacA*
1	0.5	1	2	≤0.003	*qacB*
1	1	1	2	4	–
S. aureus ATCC 6538	1	1	8	≤0.003	–

BAC, benzalkonium chloride; CHA, clorhexidine acetate; EtBr, ethidium bromide; MICs, minimum inhibitory concentrations; MRSP, methicillin-resistant *Staphylococcus pseudintermedius*; MSSP, methicillin-resistant *S. pseudintermedius*; TCL, triclosan.

In this study, we compared the *in vitro* efficacy of four commercial biocides using the methodology recommended by the SCENIHR.[25] At the same time we performed the determination of the biocide MICs to detect decreased susceptibility related to efflux activity. Only one strain showed higher EtBr MIC values compared to the wild-type *S. aureus* ATCC6538 and harboured a *qacA* gene. The *qacB*-positive MSSP strain (FMV750/10) did not show any decreased susceptibility related to efflux activity. The same strain also had a *lnu*(A) gene but was not clindamycin-resistant. This could indicate a failure in the regulation and/or induction mechanism of these genes. However, further studies are needed to address this issue. To the best of our knowledge, this is the first description of *qacA* and *qacB* genes among *S. pseudintermedius* strains.

The efficacy of chlorhexidine has been previously tested *in vitro* and also *in vivo*. In our study we found an MIC value of 1 mg/L for all MRSP and MSSP strains, which is lower than the MIC range found by Valentine and colleagues[29] (4–16 mg/L) but within the range found by Murayama *et al.*, (0.5–1 mg/L).[20] This latter study could not detect any *qacA/B* or *smr* genes. This MIC of 1 mg/L is lower than the clinically used concentrations and so it is not surprising that Otodine® and Clorexyderm Spot Gel® were efficient at killing the MSSP and MRSP strains. There was no difference in the efficacy of the chlorhexidine products, but previous studies have suggested that products with higher concentrations of CHA (3%–4%) were more effective than products with a lower concentration (2%–2.5%).[15,33] Still, an *in vivo* study comparing the use of two different chlorhexidine formulations (CHA 2% and chlorhexidine gluconate 4%) for the treatment of cephalexin-resistant *S. pseudintermedius* pyoderma found no differences in the efficacy of the two shampoos.[21]

QAC efflux pumps are known to extrude BAC;[8] however, the MICs previously found in other studies are still below the typically used concentrations of 10 g/L.[29] In our study we

detected two strains harbouring *qac* genes but their MICs were also below the in-use concentration. However, even if the strains appeared susceptible *in vitro* in the presence of these genes, they may challenge biocide therapy *in vivo*.

None of the strains with high MIC to TCL carried the newly described plasmid-mediated TCL resistance gene *sh-fabI*.[6] The higher MIC values in the two MSSP strains, and the absence of the *sh-fabI* gene may indicate that another mechanism is present, probably mutations in the original *fabI* gene, which have been previously described in *S. aureus* and *S. haemolyticus* strains.[6] A recent study assessed the MIC of TCL against MRSP and MSSP strains.[29] The authors concluded that TCL demonstrated excellent activity against all bacterial isolates with a MIC ≤ 0.5 mg/L.[29] In this study, we detected one MSSP strain with a MIC of 4 mg/L to TCL, which is higher compared to the wild-type *S. aureus* ATCC6538 (MIC ≤ 0.003) and the other *S. pseudintermedius* strains. However, when testing the bactericidal activity of Dermocanis Piocure-M,® a commercial product with a TCL concentration 750 times higher than the MIC, no bacterial growth was observed. Likewise, the MSSP strain presenting an efflux phenotype, when challenged with three commercial products containing biocides was also not able to survive. This could mean that although some strains have efflux mechanisms to biocides, they will not be able to survive if the biocides are used at the correct concentration and exposure time.

Skingel® is an antiphlogistic product, containing zinc oxide, which is known to have antibacterial properties.[26] Zinc oxide has been shown to reduce *S. aureus* viability and biofilm formation when incorporated as a nanoparticle into films of polyvinyl chloride (endotracheal tubes and catheters).[26] However, Skingel was not able to achieve a five-log reduction in the bacterial cell number and so had no bactericidal effect on *S. pseudintermedius* strains. Zinc resistance has been detected in *S. aureus* strains of animal origin and has been strongly associated with methicillin resistance.[5] Further studies are needed to evaluate heavy metal resistance in *S. pseudintermedius*.

S. pseudintermedius have become a serious therapeutic challenge and new MRSP lineages are emerging in several countries, including Portugal. Although multidrug-resistance is more common in methicillin-resistant strains, some of the methicillin-susceptible strains also exhibited multidrug-resistance profile. The use of biocides, like CHA and TCL, seems to be a clinically effective and a safe topical therapeutic option.

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